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Detection of autonomous replicating sequences (*ars*) in the genome of Epstein–Barr virus

(cloned virus DNA/Epstein-Barr virus plasmid/origin of replication)

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ABSTRACT **Epstein–Barr virus (EBV) DNA was analyzed for the presence of autonomous replicating sequences (designated *ars*) in a eukaryotic system consisting of a uracil auxotroph of *Saccharomyces cerevisiae*, YNN27, and a pBR322 hybrid plasmid, YIp5, containing the yeast uracil gene but apparently lacking a eukaryotic origin of replication. Cloned EBV DNA *Eco*RI restriction fragments, A, B, and DIJ_{het}, were judged to function in this capacity by their ability to convert YNN27 cells to the uracil phenotype after transformation with each EBV-specific fragment ligated into YIp5. Additional analyses to confirm and to specify further the location of the *ars* were performed by cleavage of *Eco*RI fragments A and B into smaller *Bam*HI fragments, which were subsequently cloned in YIp5 and tested for their ability to function as *ars*. *Bam*HI fragment X, obtained from *Eco*RI fragment A, and *Bam*HI fragment R, obtained from *Eco*RI fragment B, showed *ars* behavior. The successful recovery of the appropriate virus DNA segments in plasmid form from transformed yeast cells and the ability of these yeast cells to be propagated further substantiated the *ars* capability of the three EBV fragments.**

The plasmid form of the Epstein–Bar virus (EBV) genome is a covalently closed circular molecule that exists only intracellularly. Since its discovery, an understanding of the mechanism whereby the EBV plasmid is maintained in cells has remained an attractive but elusive objective (1, 2). The replication of the EBV plasmid is strictly regulated in synchrony with the cell cycle; the plasmid replicates only during the S phase, and the copy number is constant and remains relatively low (3). The plasmid is situated in the nucleus and exhibits a nucleosomal arrangement (4). Circular EBV genomes are found both in cell lines and in fresh tissue samples of Burkitt lymphoma and nasopharyngeal carcinoma (5). The plasmid is the only form of the EBV genome found in nonvirus-producing cell lines, but plasmids are also found in the nonproductive cellular fraction of virus-producing lines (6).

One of the intriguing aspects of EBV plasmids is that they are believed to be replicated by host DNA polymerase rather than virus-induced DNA polymerase (2). The viral enzyme is responsible for replication of the linear form of the EBV genome, which is encapsidated in virions; the enzyme is not detectable in the nonvirus-producing Raji cell line (7). In addition, the linear form of the EBV genome replicates independently of the host-cell cycle, and the relative number of virus genomes fluctuates over a wide range (8). The controlled, limited replication of the EBV plasmids, together with the absence of viral polymerase, suggests that this EBV DNA form is replicated by host DNA polymerase.

The existence of specific sites at which the genomes of numerous prokaryotes and several eukaryotic viruses initiate DNA synthesis is accepted fact, and these sites have been termed origins of replication (9). However, in eukaryotes the existence of such defined origins of replication, while assumed, has not been demonstrated clearly except in a limited number of small extrachromosomal DNAs such as mitochondrial DNA in which initiation occurs at only one site for the entire molecule or from each strand (9, 10). Cellular and complex viral genomes (e.g., herpesviruses) may have some similarities in their replication; therefore, viruses of this type may provide a more useful system in which to study eukaryotic-like origins of replication. The EBV plasmid in particular is likely to have and indeed may be the best candidate to contain eukaryotic origins (2).

Recent advances in molecular cloning have produced a system in yeast analogous to replication systems in prokaryotes that can be utilized to screen eukaryotic DNA segments for their ability to replicate autonomously (11–13). Stinchcomb *et al.* (14) first demonstrated the existence of eukaryotic DNA sequences that could replicate in such a system. To date, several sources of eukaryotic DNAs have been found to contain sequences that can replicate in this yeast system. Among these are *Saccharomyces cerevisiae*, *Neurospora crassa*, *Dictyostelium discoideum*, *Drosophila melanogaster*, *Zea mays*, and mitochondrial DNA. Those DNA sequences that replicate in this manner have been referred to as autonomously replicating sequences (designated *ars*) and are presumably functional eukaryotic origins of replication (13). Several yeast genes coding for various amino acids and DNA precursors paired with their corresponding yeast auxotrophs have been isolated and used as the basic selective system to analyze for the presence of *ars* in eukaryotic DNAs.

One of these systems utilizes the *URA3* gene of *S. cerevisiae* for selection (13). The plasmid of interest, YIp5, is a hybrid plasmid consisting of pBR322 and the *URA3* gene of *S. cerevisiae*. Therefore, YIp5 can be utilized in a yeast selective system and can be grown and recovered in an *Escherichia coli* system as well. In addition, YIp5 lacks a functional yeast origin of replication and cannot transform the corresponding *URA* auxotroph of *S. cerevisiae* unless an exogenous *ars* is incorporated into the plasmid. This system then provides the necessary screening system for the detection of *ars* within unrelated DNAs. Recent work from several laboratories has shown the validity of this yeast vector system when used in this capacity (11–13, 15). We have used this selection system in experiments designed to determine the number and location of *ars* in the genome of EBV.

MATERIALS AND METHODS

Source of EBV DNA. The preparation of bacterial clones of virus DNA from the W91 strain of EBV has been described elsewhere (16). The subsequent growth, isolation, and purification

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Abbreviation: EBV, Epstein–Barr virus.

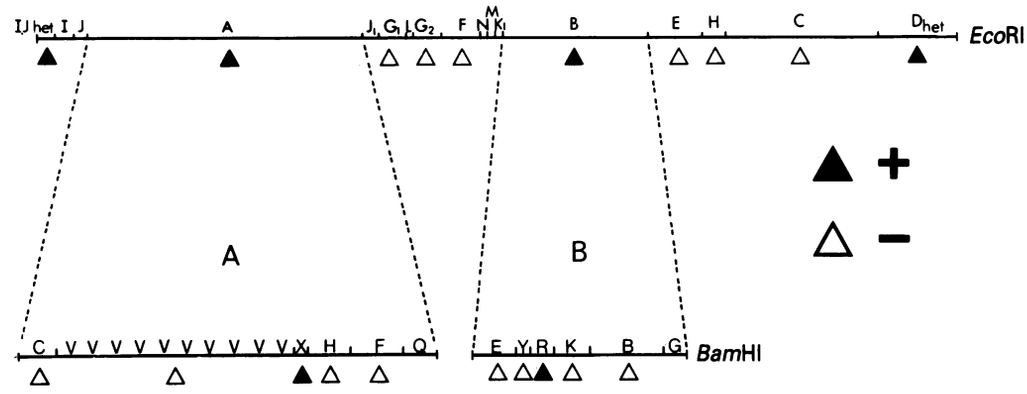

FIG. 1. Identification of *Eco*RI and *Bam*HI fragments of EBV DNA (W-91 DNA) that contain *ars*. The fragments were tested in the YIp5–YNN27 system for their ability to function as *ars*. ▲, Fragments containing *ars*; △, fragments tested but not containing *ars*.

of the various genomic fragments of EBV used in this study have been described in detail (17).

Yeast Strain and Vector. The uracil auxotroph of *S. cerevisiae*, YNN27, and the hybrid yeast plasmid YIp5 were generously supplied by Dr. Ron Davis of Stanford University. The conditions used in the growth and maintenance of YNN27 have been described (13).

Cloning of EBV DNA into YIp5. YIp5 contains single-cut sites for the restriction enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Sal* I and resistance genes for both tetracycline and ampicillin (13). The cloning procedures used for this vector involved cleavage of the EBV DNA and the YIp5 with the desired restriction enzyme, followed by rehybridization of the mixture of DNA molecules and ligation of the hybrid molecules with T4 ligase. After the cells were made competent by the $CaCl_2$ technique (18) and *E. coli* strain HB101 was transformed, selection of vector-containing inserts could be done on medium containing either tetracycline or ampicillin. Detection of the resultant clones containing EBV DNA inserts was done by *in situ* hybridization with the appropriate labeled DNA probe (19). Subsequent growth and recovery of the hybrid plasmids was accomplished by the standard methods (17).

Transformation of Yeast. DNA transformation of *S. cerevisiae* was done in a manner essentially identical to that used in the transformation of bacteria except for an initial digestion step to convert the yeast cells to spheroplasts (12). The yeast cells were then plated on minimal medium without uracil in the presence of sorbitol. YNN27 transformants to the URA^+ phenotype were recognized by the production of colonies after several days; these transformants can be quantitated as the number of colonies per μg of input DNA (13).

Transformation of Bacteria. The method used for the transformation of the *E. coli* strain HB101 was essentially that described by Dagert and Ehrlich (18).

Isolation of EBV DNA-Containing Plasmids from Yeast. YNN27 cells were incubated with Zymolyase (Kirin, Japan) at a final concentration of 1 mg/ml in 1 M sorbitol/4 mM EDTA for 1 hr at 30°C and subsequently lysed by osmotic shock. The lysate was then incubated with proteinase K (final concentration, 100 μg/ml) at 45°C for 30 min. The total yeast DNA was then precipitated by addition of ethanol and subsequently used to transform *E. coli* strain HB101.

Detection of DNA by Hybridization. DNA samples of interest were cleaved with the desired restriction enzyme, sep-

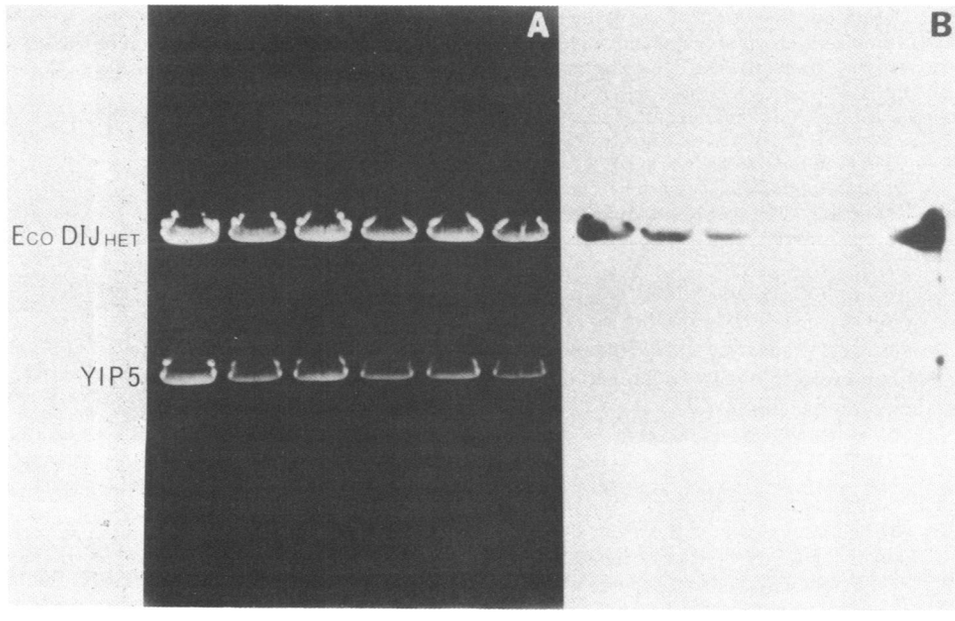

FIG. 2. Recovery of *Eco*RI fragment DIJ_{het} from transformed yeast cells. YNN27 cells were transformed with the *Eco*RI DIJ_{het} fragment as described. Total DNA was isolated and subsequently used to transform *E. coli* strain HB101. The resultant plasmids were analyzed by Southern blot analysis with purified ^{32}P-labeled EBV *Eco*RI DIJ_{het} fragment as probe. (A) Ethidium bromide-stained agarose gel of representative plasmids cleaved with *Eco*RI restriction enzyme. (B) Autoradiogram of blot-hybridization analysis of representative plasmids with labeled DIJ_{het} fragment as probe.

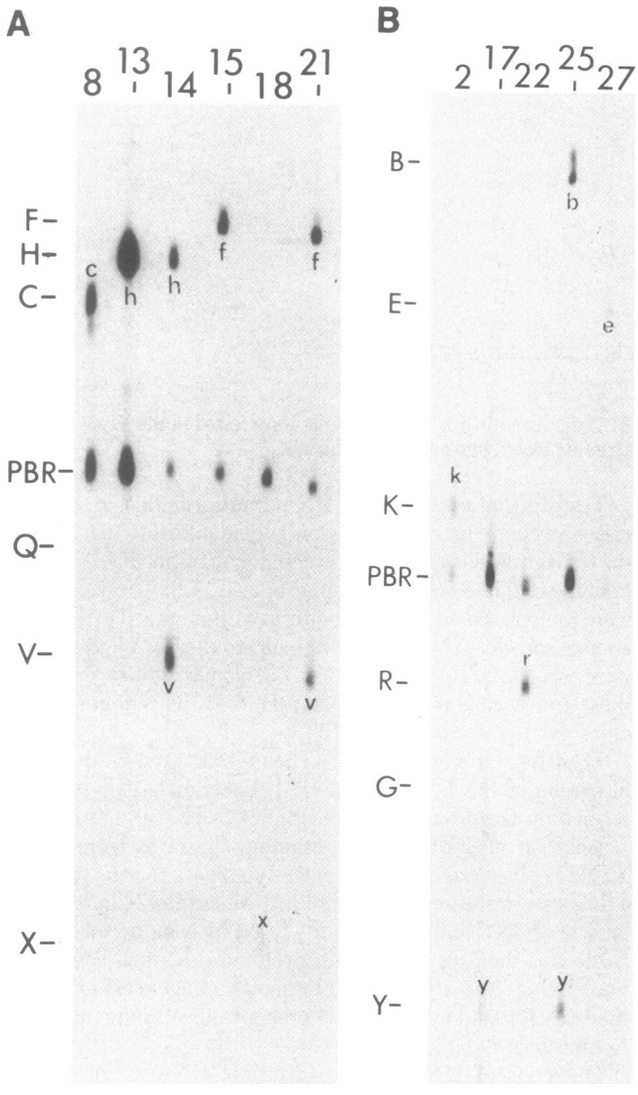

FIG. 3. Determination of specific *Bam*HI fragments present in clones of *Eco*RI A (*A*) and B (*B*) fragments. EBV *Eco*RI A and B fragments were cleaved with *Bam*HI restriction enzyme, ligated into *Bam*HI-digested YIp5, and used to transform *E. coli* strain HB101. Purified plasmid DNA was labeled with [^{32}P]dCTP by nick-translation and subsequently hybridized to Southern blots of *Bam*HI-digested EBV *Eco*RI fragment A or B. Specific *Bam*HI fragments are designated by their appropriate letter (see Fig. 1). (*A*) *Bam*HI clones hybridized to blots of *Bam*HI-digested *Eco*RI fragment A. (*B*) *Bam*HI clones hybridized to blots of *Bam*HI-digested *Eco*RI B fragment. pBR, bacterial cloning vector present in all samples.

arated by agarose-gel electrophoresis, and blotted onto nitrocellulose as described by Southern (20). Hybridization conditions and visualization of the resultant bands by autoradiography have been described. Labeled probes of interest were prepared with [^{32}P]dCTP (ICN) by the method of Rigby (21).

RESULTS

Screening of the EBV Genome for the Presence of *ars*. The initial screening of the virus genome involved the use of MUA3 clones of EBV DNA *Eco*RI fragments that were ligated into *Eco*RI-digested YIp5; these clones were used to transform YNN27 and were observed for their ability to function as *ars* as judged by the production of URA$^+$ yeast colonies. EBV *Eco*RI fragments A, B, C, DIJ$_{het}$, E, F, G, G$_2$, and H, which comprise greater than 95% of the virus genome, were assayed by this method (Fig. 1). Three DNA segments, A, B, and DIJ$_{het}$, were found to function as *ars* by the successful conversion of YNN27 URA$^-$ to the URA$^+$ phenotype; the remaining *Eco*RI fragments were determined to be negative based upon this criterion.

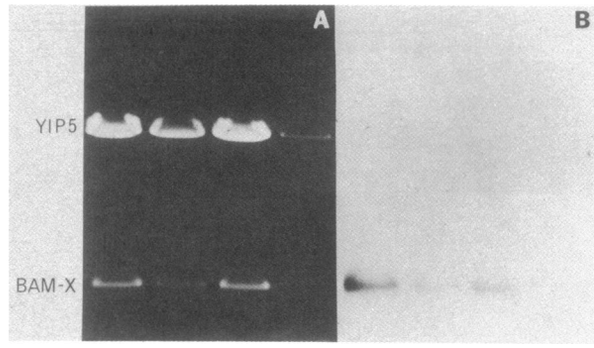

FIG. 4. Recovery of *Bam*HI fragment X from transformed yeast cells. YNN27 cells were transformed with the BamHI fragment X, and total DNA was isolated and subsequently used to transform *E. coli* strain HB101. The resultant plasmids were analyzed by Southern blot analysis with purified ^{32}P-labeled EBV *Bam*HI fragment X as probe. (*A*) Ethidium bromide-stained agarose gel of representative plasmids cleaved with *Bam*HI restriction enzyme. (*B*) Autoradiogram of blot hybridization analysis of representative plasmids using labeled *Bam*HI fragment X as probe.

Demonstration of EBV DNA in Transformed Yeast. That the hybrid YIp5–DIJ$_{het}$ plasmid was present in the YNN27-transformed cells was demonstrated directly by recovery of such plasmids after transformation of *E. coli* with total yeast DNA isolated from the URA$^+$ cells. Fig. 2 shows agarose gels of *Eco*RI-cleaved YIp5–DIJ$_{het}$ plasmids obtained by this method and the subsequent blot hybridization of the ^{32}P-labeled DIJ$_{het}$ to the DNA fragments, indicating that the DIJ$_{het}$ fragment is indeed present.

Location of *ars* Present in EBV Fragments A and B. As mentioned above, transformation of yeast with YIp5 plasmids containing either fragment A or B resulted in the production of URA$^+$ colonies, but the two fragments were of such a size that the relative location of the *ars* was still unclear. This problem was bypassed by the use of a second enzyme, *Bam*HI, which cleaved both *Eco*RI fragments A and B into six smaller fragments each (see Fig. 1) and allowed ligation of the resultant fragments into YIp5 (Fig. 3). Subsequent transformation of YNN27 cells with

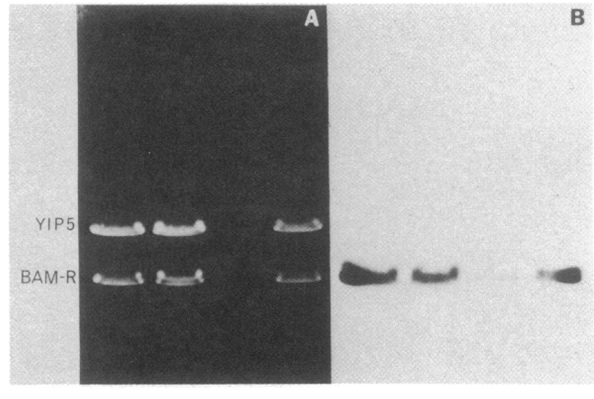

FIG. 5. Recovery of *Bam*HI fragment R from transformed yeast cells. YNN27 cells were transformed with *Bam*HI fragment R, and total DNA was isolated and subsequently used to transform *E. coli* strain HB101. The resultant plasmids were analyzed by Southern blot analysis with purified ^{32}P-labeled EBV *Bam*HI fragment R as probe. (A) Ethidium bromide-stained agarose gel of representative plasmids cleaved with *Bam*HI restriction enzyme. (B) Autoradiogram of blot hybridization analysis of representative plasmids with labeled *Bam*HI fragment R as probe.

Table 1. Transformation of URA$^-$ yeast (YNN27) with clones of EBV DNA*

EBV DNA clone	Time, days 3	5	8	Colonies per μg of DNA,†
*Eco*RI fragment A‡				
8(C)	−	−	−	0
13(H)	−	−	−	0
14(H,V)	−	−	−	0
15(F)	−	−	−	0
18(X)	+++	+++	+++	8,500
21(F,V)	−	−	−	0
*Eco*RI fragment B‡				
2(K)	−	−	−	0
17(Y)	−	−	−	0
22(R)	+++	+++	+++	8,200
25(B,Y)	−	−	−	0
27(E)	−	−	−	0
DIJ_{het}	+++	+++	+++	9,200

* YNN27 cells were transformed by each EBV DNA YIp5 plasmid. The number of URA$^+$ YNN27 colonies was determined each of the times indicated in column one for the number of colonies per μg of DNA for each EBV DNA clone. The specific EBV DNA fragments are designated by the appropriate letters (in parentheses in column one) as shown in Fig. 1.

† The quantitation of colonies is based upon the number of colonies observed after plating and the ability of the resultant colonies to be propagated upon restreaking; abortive colonies were not scored as positive.

‡ Digested with *Bam*HI.

each of the purified plasmids revealed one *ars*-containing *Bam*HI fragment located in *Eco*RI fragment A and one in *Eco*RI fragment B. As indicated in Fig. 1, these fragments were *Bam*HI X (present in *Eco*RI fragment A) and *Bam*HI R (present in *Eco*RI fragment B). The transformation frequency of *Bam*HI fragment X was 8,500 colonies per μg of input DNA and for *Bam*HI fragment R was 8,200 colonies per μg of input DNA (see Table 1). Transformation attempts with the remaining hybrid plasmids resulted in the production of either no colonies or, in some cases, various low levels of colonies, which proved to be abortive transformants as evidenced by their inability to be propagated. However, transformants produced by plasmids containing *Eco*RI DIJ_{het}, *Bam*HI X, or BamHI R fragment could be propagated upon restreaking and, therefore, were judged to act as true *ars*.

Recovery of *Bam*HI Fragments from Transformed YNN27. Total yeast DNA from URA$^+$ yeast cells 4 days after transformation with *Bam*HI fragments X and R was used to transform HB101 under ampicillin selection. Figs. 4 and 5 show agarose gels of *Bam*HI-digested plasmids recovered by this method and the subsequent blot hybridization of the fragments with the use of purified ^{32}P-labeled *Bam*HI X and R that revealed the presence of the fragments in question.

Location and Quantitation of EBV Autonomous Replicating Sequences. Fig. 1 and Table 1 summarize the results obtained in this study. As shown schematically in Fig. 1, the EBV genome contains three segments that act as *ars* in this yeast system: *Eco*RI DIJ_{het}, *Bam*HI X (in *Eco*RI fragment A), and *Bam*HI R (in *Eco*RI fragment B). All other EBV DNA fragments, both *Eco*RI and *Bam*HI fragments, failed to transform YNN27 cells to the URA$^+$ phenotype and, therefore, presumably do not function as *ars* in this system. Table 1 quantitates the three positive EBV fragments on the basis of colonies produced per μg of input DNA.

DISCUSSION

Latency is the hallmark of herpesvirus infections, and the EBV plasmid has been proposed to be possibly the molecular basis of this biologic feature (5). So far, plasmid forms have been demonstrated in several other herpesvirus systems in addition to EBV (e.g., *Herpesvirus* samiri; ref. 22). The role of this molecular form in these infections and in relation to the other diseases that arise after exposure to EBV, such as Burkitt lymphoma and nasopharyngeal carcinoma, is unknown. For example, whether or not the plasmid is transcribed and expressed in these conditions remains unexplored (5).

Experiments of the type described above revealed the presence of three defined virus DNA segments that could function as an *ars* in this system. These three EBV fragments, *Eco*RI DIJ_{het}, *Bam*HI X, and *Bam*HI R, all satisfied the necessary criteria to be classified as *ars*; the high frequency of production of transformed yeast colonies, the successful recovery of EBV DNA-containing plasmids from transformed yeast cells, and the ability of transformed yeast colonies to be propagated confirmed the identifications.

*Eco*RI DIJ_{het} is a cloned fragment comprising the fused termini of the linear DNA molecule and is found in this form naturally only in the plasmid form of the virus genome. This fragment consists of a variable number of direct tandem repeats of a 0.45-kilobase segment of DNA and a stretch of unique DNA sequences. *Bam*HI fragments X and R, in contrast, are contained within the interior of the molecule in both the linear and plasmid forms of the EBV genome. The fact that these three fragments are recognized as *ars* is of special importance because of their apparent association with other important functions of the virus genome.

In the case of *Bam*HI X, a transcript is generated from this fragment in several EBV-transformed lymphoblastoid cell lines (23) that indicates the presence of an RNA polymerase recognition site. This observation is most interesting because of the recent hypothesis that points of DNA replication are related to units of transcription (9). Also Hayward and co-workers (24) have shown that *Bam*HI fragment X contains sequences that are homologous to mammalian cell DNA sequences, although the significance of this observation is unknown at this time. Likewise, in some of these cell lines, stable polyribosomal transcripts are found that originate from both the left- and right-hand DNA sequences immediately adjacent to *Bam*HI fragment R (including *Bam*HI fragments Y, K, and B) and that have been associated also with the generation of nonstandard or defective virus genomes isolated from several EBV-transformed cell lines. These observations are of interest due to the close relationship between origins of replication and the production of defective virus genomes (25). These correlations, while certainly not conclusive, do lend support to the status of *Bam*HI fragments X and R as *ars*. Likewise the DIJ_{het} fragment has been implicated in the generation of defective DNA and also serves as a template for the production of abundant RNA transcripts.

It should be stated at this point that, even though the abovementioned theories pertaining to the *ars* found in the EBV plasmid are feasible, the actual role of these fragments in the replication process is unknown and cannot be determined with any degree of certainty based solely upon the above experiments. However, these results have allowed us to establish the possible locations of origins of DNA synthesis. In order to resolve this question, we have initiated work that should provide the necessary authentication of the role of these *ars* in mammalian cells. The most important aspect of the experiment will be to determine if those EBV DNA sequences containing putative origins of replication will allow a stable extrachromosomal form of the virus DNA to become established. The results of such experiments should help to provide answers to the question of how the EBV plasmid replicates and is maintained in cells.

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